

## **E.7**



## **IN Vitro Adverse Drug Effects (ADE) Screening Assays**

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## **In vitro ADE screening**

- Early screening of ADE to aid selection of drug candidates with diminished safety liability

## Adverse Drug Effects to avoid

- CYP3A inhibition and induction
- Hepatotoxicity
- Non-hepatic organ toxicity

## Critical technologies for ADE screening

- Pre-pooled cryopreserved human hepatocytes
- Plateable cryopreserved human hepatocytes
- High throughput CYP3A4 assay

## **Pre-POOLED CRYOPRESERVED HUMAN HEPATOCYTES- Comparison with Single Donor Hepatocytes**

**Pooled hepatocytes allow the generation of data  
representing a "normalized" human population**

- Important for routine screening of ADMET drug properties

**Single donor hepatocytes provide data allowing  
evaluation of individual differences**

- Important for enzyme induction studies (e.g. FDA requires  
N=3)

## **Pre-pooled cryopreserved human hepatocytes (HuP)**

**Select human hepatocytes isolated and  
cryopreserved from 5 male and 5 female  
donors**

**Thaw and refreeze to constitute HuP**

## Effects of Pooling on CYP Activities

Donor ID	CYP1A2	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A4
HuP40	106	6.60	133.5	18.60	33.5	652
Theoretical	139.4	5.4	100.1	13.3	29.1	310.7

Pooling did not compromise DME activities

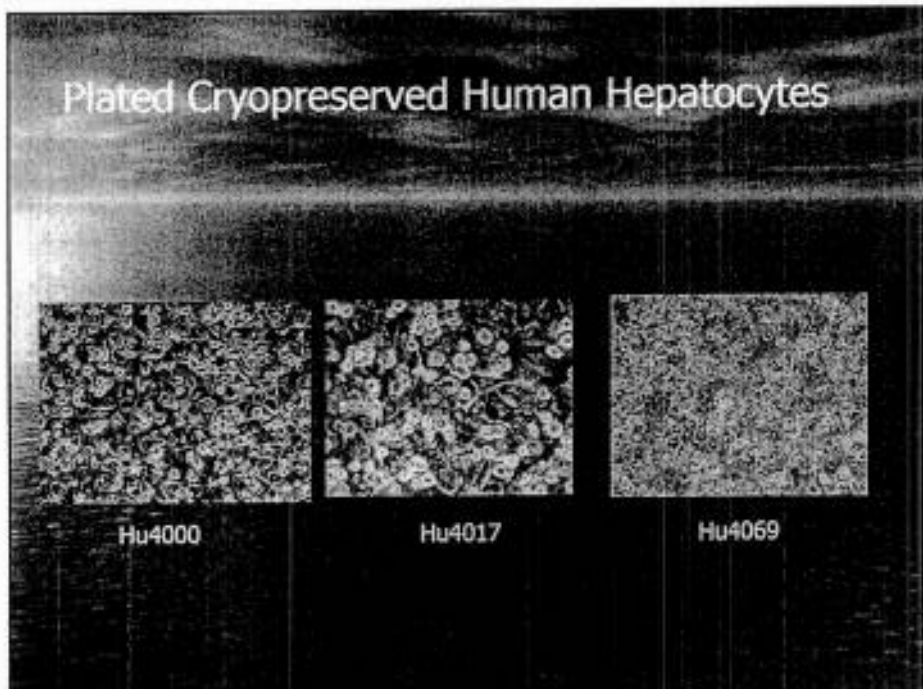
\*calculated average of activities of the 10 lots of hepatocytes (5 male; 5 female) used for pooling

Plateable cryopreserved  
human hepatocytes

## Cryopreserved Human Hepatocytes

Lot #	Yield (cells/vial)	Viability (trypan blue)	Plating	Confluency
HU4003	$4.5 \times 10^6$	86%	YES	100%
HU4001	$6.0 \times 10^6$	80%	NO	20%
HU4004	$6.0 \times 10^6$	80%	NO	30%
HU4000	$7.2 \times 10^6$	93%	YES	100%
HU4013	$7.3 \times 10^6$	92%	YES	75%
HU4016	$6.2 \times 10^6$	81%	YES	100%
HU4021	$5.4 \times 10^6$	89%	YES	70%
HU4022	$5.5 \times 10^6$	91%	YES	80%
HU4026	$5.85 \times 10^6$	91%	NO	10%
HU4027	$5.9 \times 10^6$	92%	NO	30%
HU4028	$3.2 \times 10^6$	83%	YES	50%
HU4023	$2.1 \times 10^6$	89%	NO	20%
HU4029	$6.0 \times 10^6$	90%	YES	80%

## Plated Cryopreserved Human Hepatocytes



## Why plateability is important

Plateable cryopreserved hepatocytes represent "higher quality" cells than nonplateable cells

Extension of applications:

- Hepatocyte in suspension dies quickly ( $T_{1/2}$  about 6 hours)

- Plated hepatocytes are viable for weeks

- Prolonged drug metabolism studies
- Cytotoxicity
- Enzyme induction
- Transporters

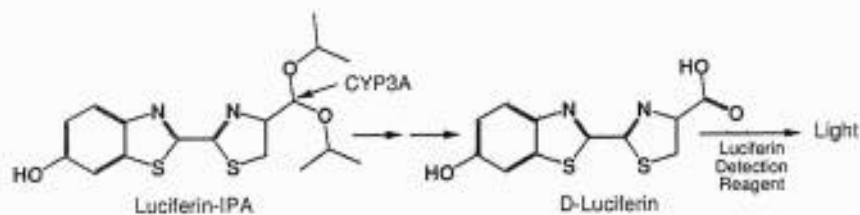
## High Throughput CYP3A4 Assay

Li, AP. Drug Metab Disp (in press)

## CYP3A4 assay

- Traditional CYP3A4 assays: testosterone 6- $\beta$  hydroxylation; medazolam 1'-hydroxylation
- The assays rely on LC/MS: costly; low throughput
- Needed: Highly specific plate reader assay for CYP3A4

## Luciferin-IPA metabolism by CYP3A4



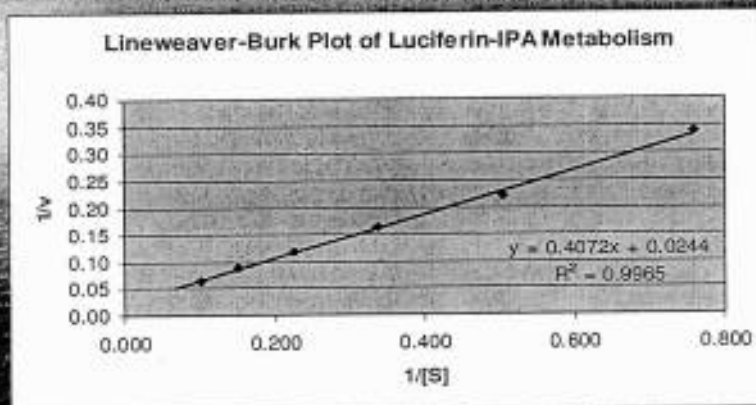
Jim Cali, Promega Inc., Personal Communication

### K<sub>m</sub> and V<sub>max</sub> Determination by Lineweaver-Burk Plot

K<sub>m</sub>: 15  $\mu$ M

V<sub>max</sub>: 41 pmol/min/million hepatocytes

HU-4065; 1 day culture

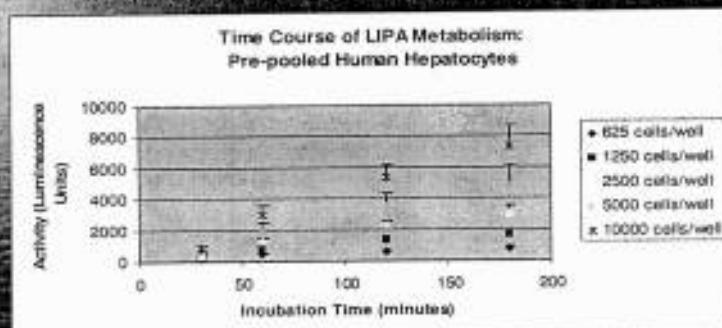


### HTS CYP3A Inhibition Screening with Pre-pooled Cryopreserved Human Hepatocytes

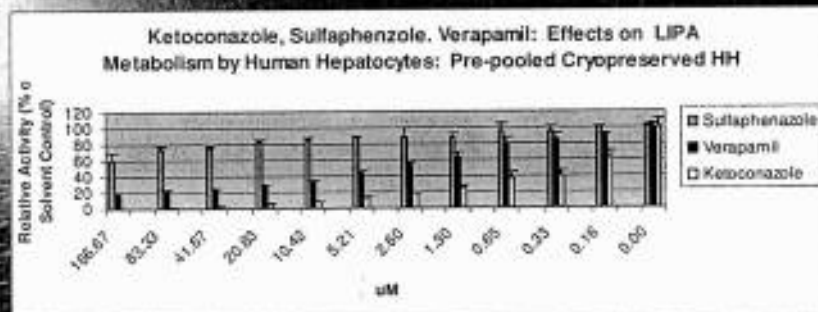
## HTS Human Hepatocyte CYP3A Inhibition

- 384-well plate format
- Pre-pooled cryopreserved human hepatocytes (Hup 79)
- LIPA metabolism as endpoint
- Robot-assisted addition of LIPA, test articles, human hepatocytes, and detection reagent

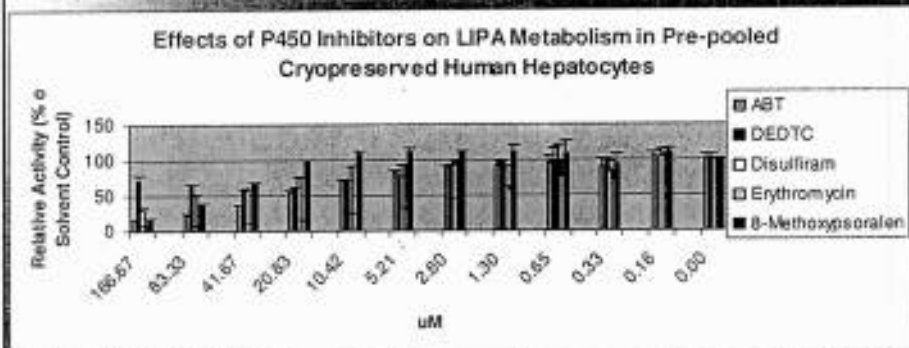
## Time-course of LIPA metabolism: Pre-pooled cryopreserved human hepatocytes



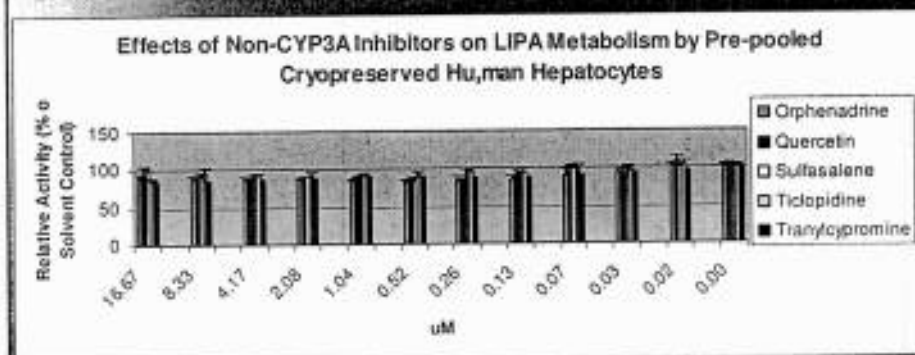
## Effects of Inhibitors



## Effects of Inhibitors



## Effects of Inhibitors



## Calculated IC50 values (uM)

Allopurinol	>16.7	Orphenadrine	>16.7
ABT	22.4	Quercetin	>16.7
DEDTC	>16.7	Quinidine	>33.3
Disulfiram	59.3	Sulfasalazine	>166.7
Erythromycin	1.3	Sulfaphenazole	>166.7
Fluoxetine	6.7	Ticlopidine	>16.7
8-methoxysoralen	51.3	Tranylcypromine	>16.7
Ketoconazole	0.052	Verapamil	2.7

## HTS CYP3A4 inhibition assay with pre-pooled human hepatocytes

Pre-pooled human hepatocytes as a physiologically more relevant equivalent of human liver microsomes

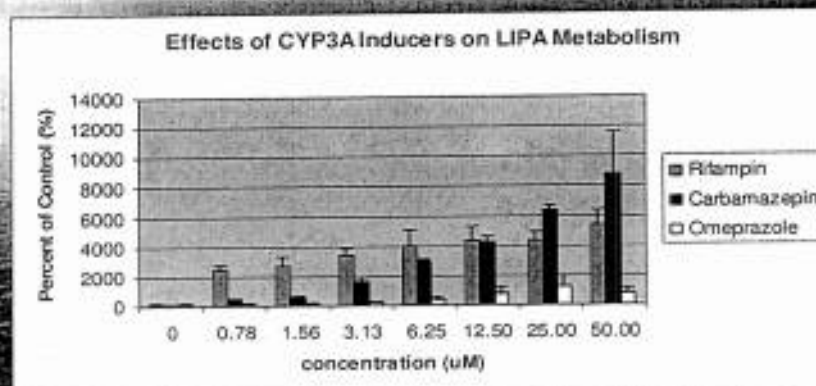
- Intact plasma membrane
- Active uptake transporters
- Uninterrupted, complete, physiological concentrations of DME and cofactors
- LIPA metabolism as a higher throughput assay for CYP3A4
  - Specificity illustrated by model P450 inhibitors

## Higher Throughput CYP3A Induction Assay

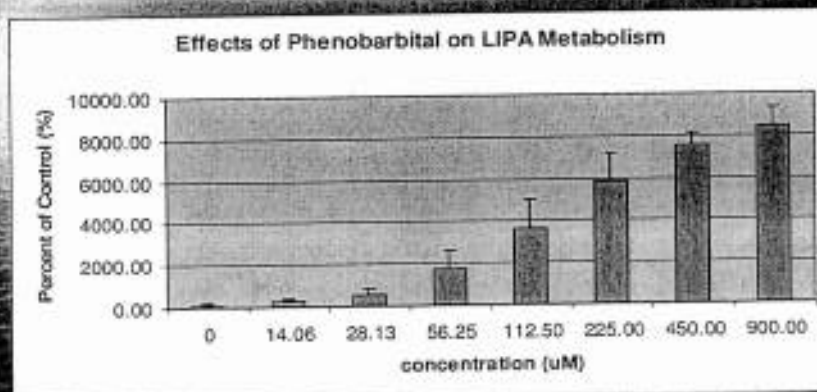
## HTS CYP3A Induction

- 96-well plate format
- 50,000 cells/well
- Three-day treatment
  - Day 1: Plate cells
  - Day 2: Overlay with matrigel™
  - Day 3: Treat with inducers
  - Day 6: CYP3A activity evaluation (in situ)
- Luciferin-IPA as CYP3A4 substrate

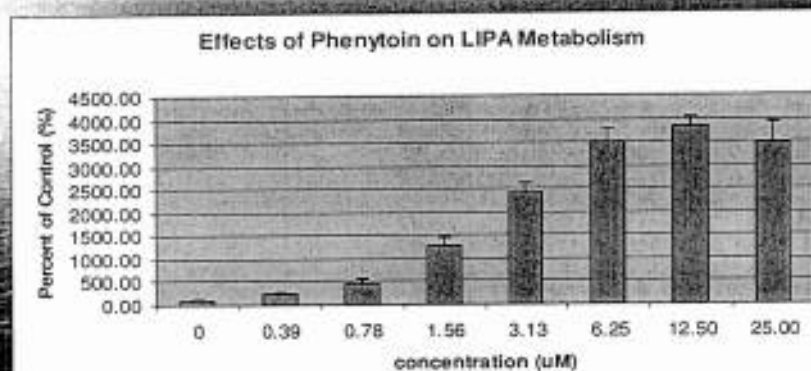
## CYP3A inducers induce LIPA metabolism (Hu4065): Rifampin, carbamazepin, omeprazole



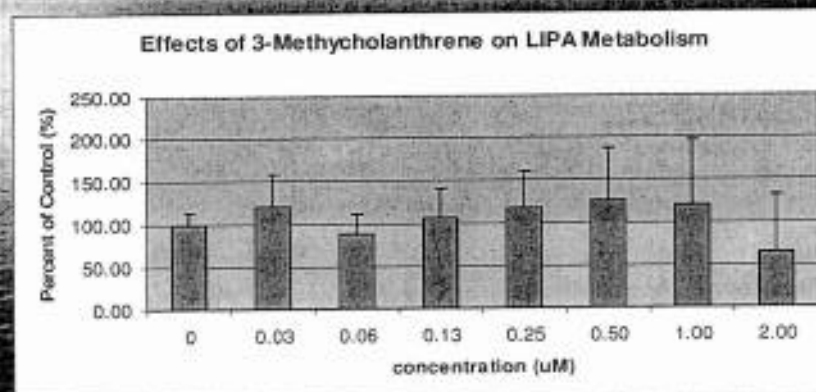
## Phenobarbital induction of LIPA metabolism (Hu4065)



## Phenytoin metabolism of LIPA metabolism (Hu4065)



## 3-MC, a CYP1A inducer, does not induce LIPA metabolism (Hu4065)



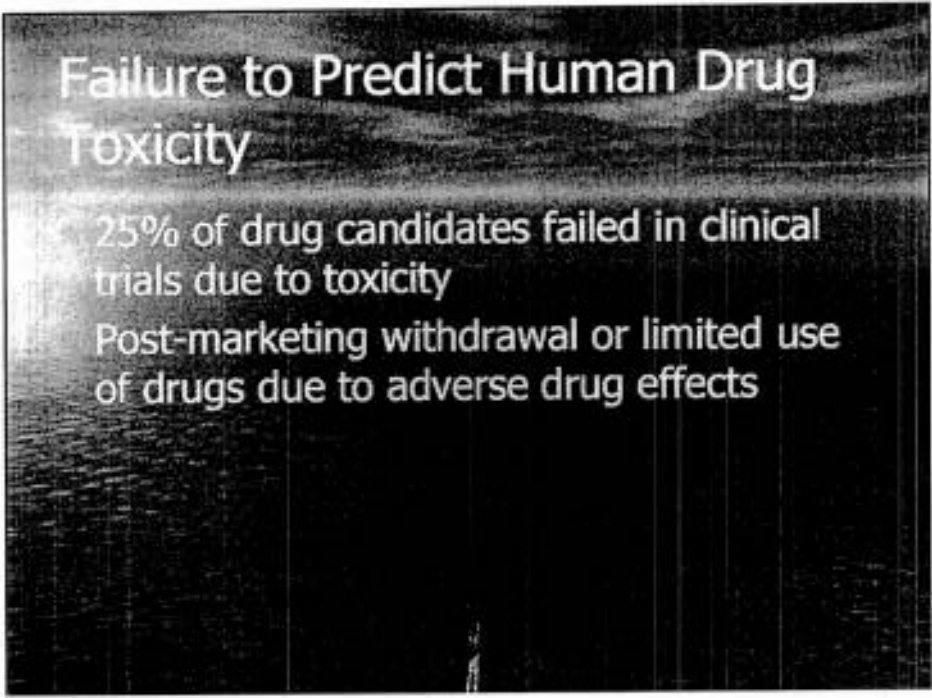
## Conclusion

Luciferin-IPA is a suitable substrate for the evaluation of CYP3A activity in human hepatocytes

- Linear time-course to up to 2 hours
- Concentration dependent metabolism following Michaelis-Menton kinetics ( $K_m$ : 15  $\mu$ M;  $V_{max}$ : 41 pmol/min/million hepatocytes)
- Inhibition observed for ABT and ketoconazole but not non-CYP3A4 selective inhibitors
- Induction observed for CYP3A but not CYP1A inducers



## Screening assays for organ-specific toxicity



## Failure to Predict Human Drug Toxicity

- 25% of drug candidates failed in clinical trials due to toxicity

- Post-marketing withdrawal or limited use of drugs due to adverse drug effects

## Hepatocyte Cytotoxicity Assay

Screening assay for acute  
hepatotoxicity  
(Li, AP (2009). ALTEX)

## Human Hepatocyte Cytotoxicity Assay

ATP/MTT/Alamar Blue endpoints as most  
routinely used

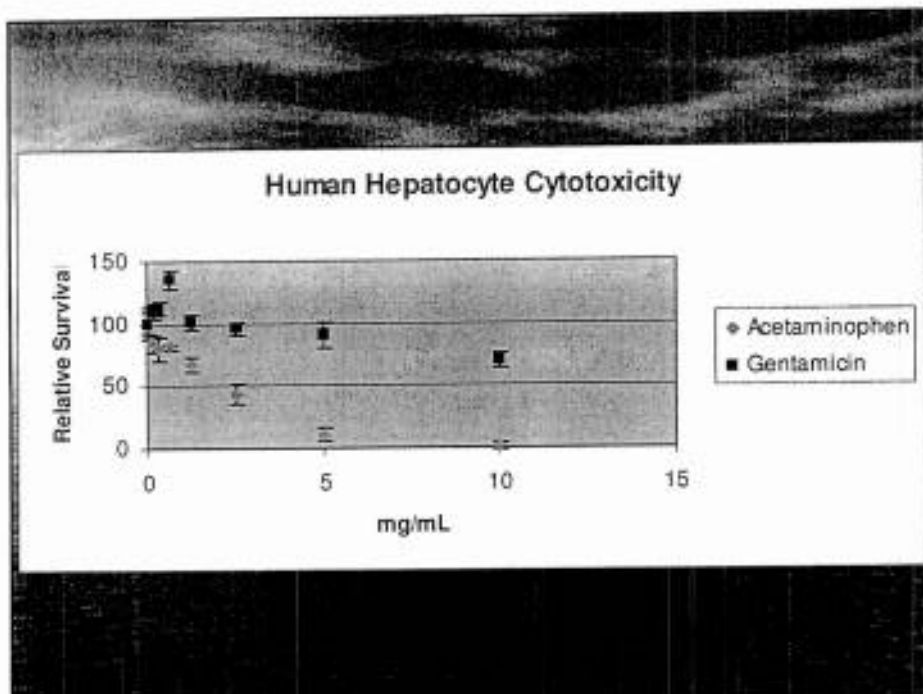
- Cryopreserved human hepatocytes in suspension/short-term (hours) treatment for HTS
- Plateable cryopreserved human hepatocytes for longer term (days) treatment

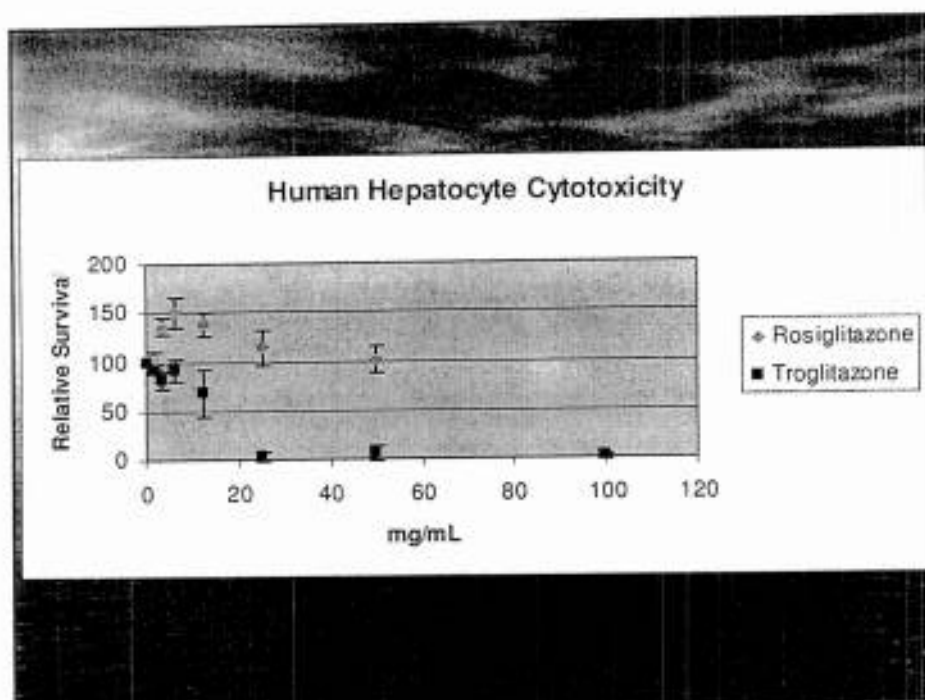
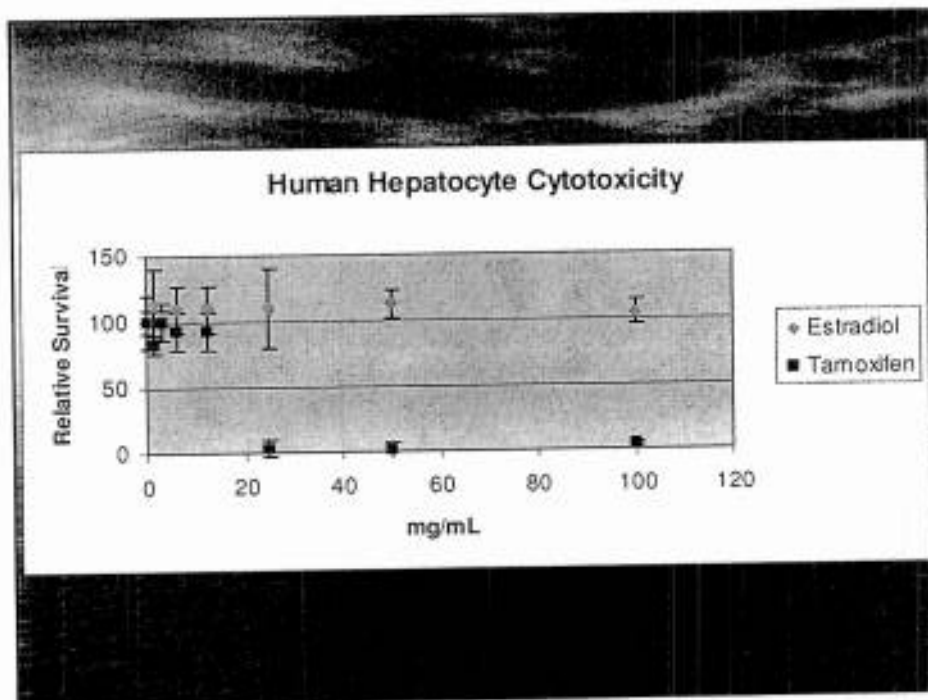
## HTS Human Hepatocyte Cytotoxicity Assay

Plateable cryopreserved human hepatocytes

384-well plate format (collagen-coated)

- Day -1, plate 1500 – 10,000 cells per well in Hepatocyte Plating Medium
- Day 0, change medium to Hepatocyte Treatment Medium containing test articles
- Day 1, ATP content quantification with ATPLite®



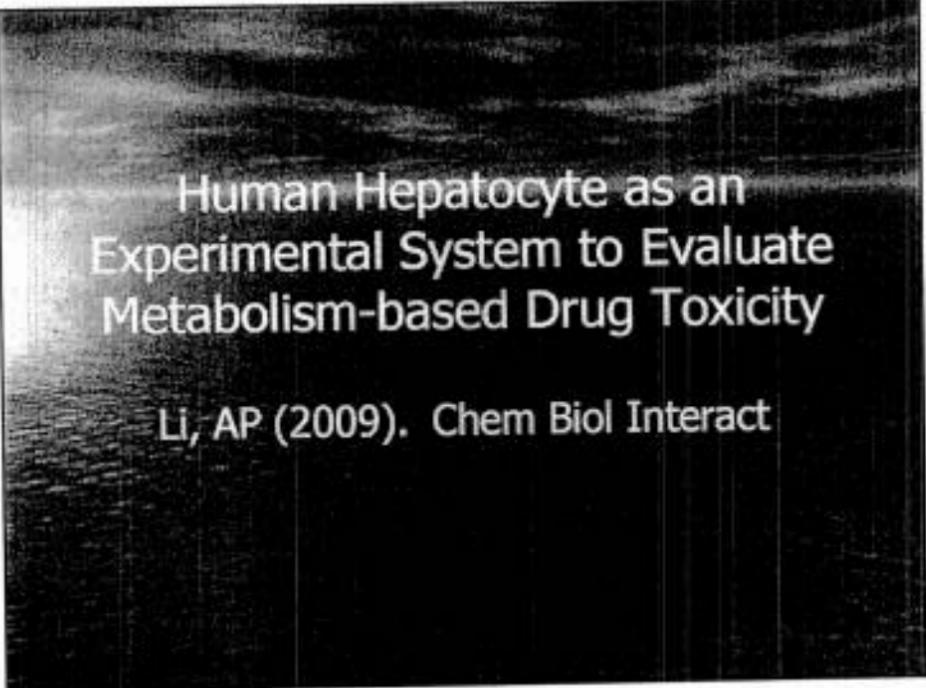


## HTS Hepatocyte Cytotoxicity Assay

- Apparent delineation of hepatotoxic and less hepatotoxic drugs
- Requires minimum (<100 ug) materials
- 24-hrs from dosing to results

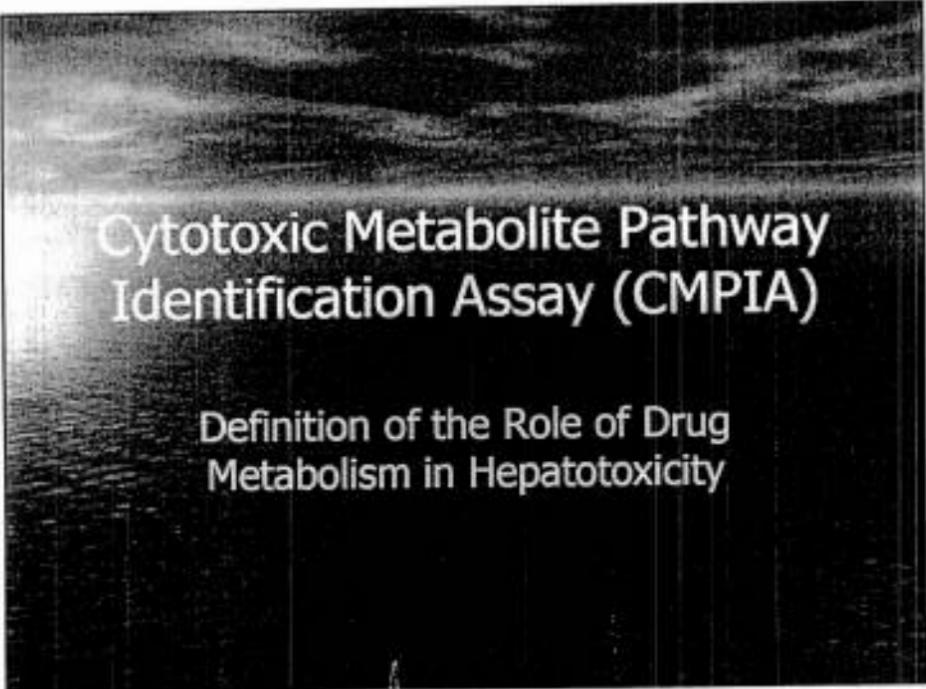
## Hepatocyte Cytotoxicity Assays

- Success depends on the choice of comparator
  - Best results if all compounds compared below to similar structural class, with a known positive compound for comparison



**Human Hepatocyte as an  
Experimental System to Evaluate  
Metabolism-based Drug Toxicity**

Li, AP (2009). Chem Biol Interact



**Cytotoxic Metabolite Pathway  
Identification Assay (CMPIA)**

Definition of the Role of Drug  
Metabolism in Hepatotoxicity

## Drug metabolism as a determinant of safety

- Organ-selective toxicity
- Species-selective toxicity
- Individual-selective toxicity

## Cytotoxic Metabolic Pathway Identification Assay (CMPIA)

Approach similar to metabolic phenotyping: used P450 inhibitors to evaluate the role of P450 metabolism in drug toxicity

## Experimental Systems

Plateable cryopreserved human hepatocytes

Chinese hamster ovary (CHO) cells, representing a metabolically incompetent nonhepatic cell system (negative control)

- ATP or MTT as endpoints

## CMPIA: Proof of Concept

Toxicant: Aflatoxin B1

Inhibitors: 1-aminobenzotriazole (nonspecific mechanism-based P450 inhibitor)

- Concept: AFB1 requires P450 metabolism to be cytotoxic

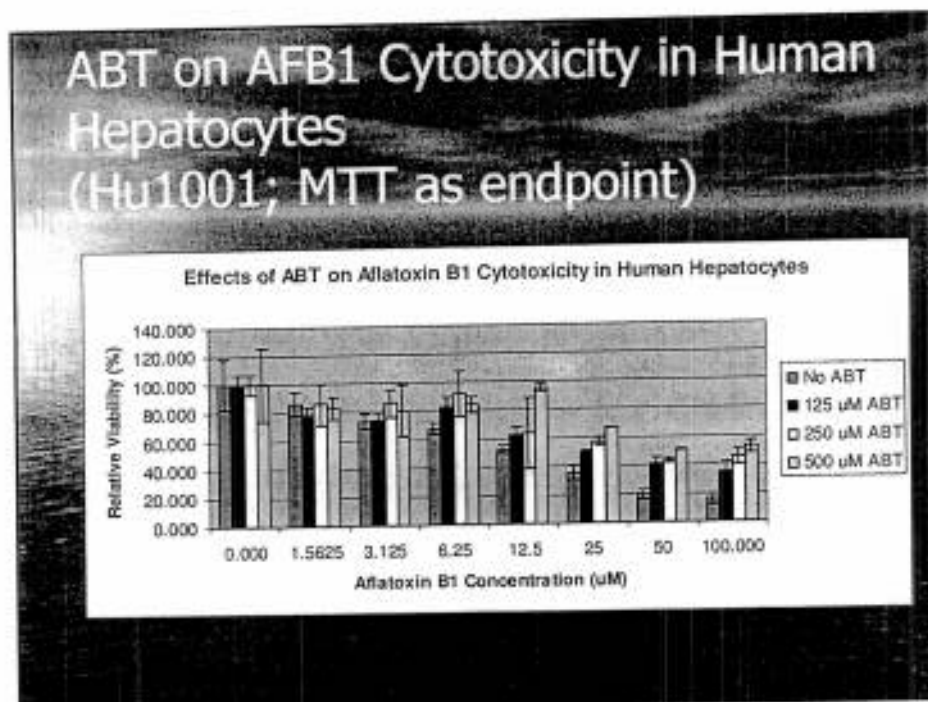
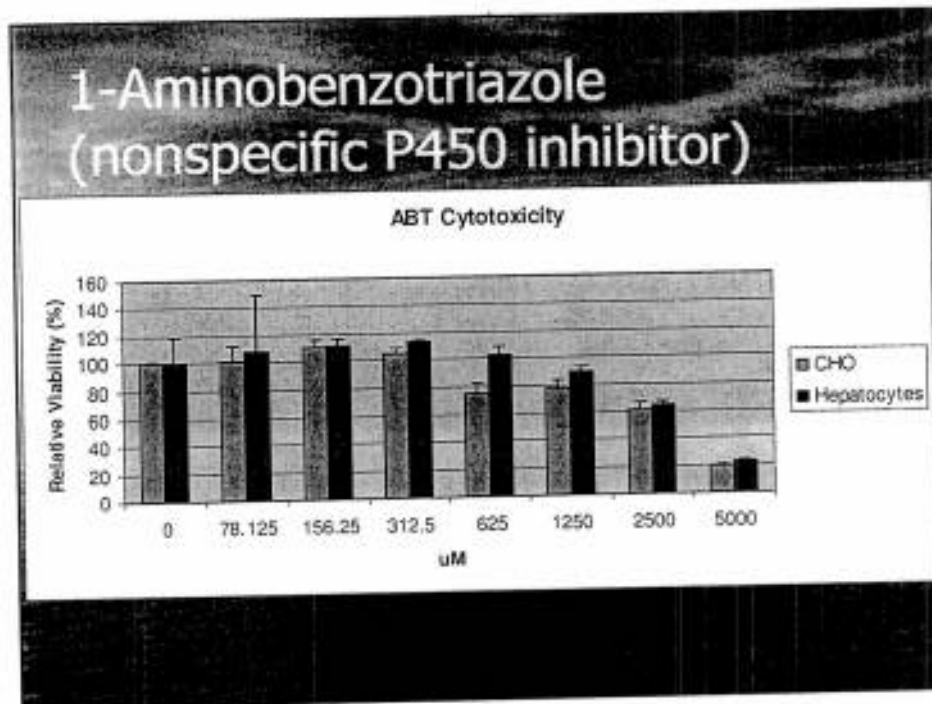
## Hepatocyte Cytotoxic Metabolism Pathway Identification Assay

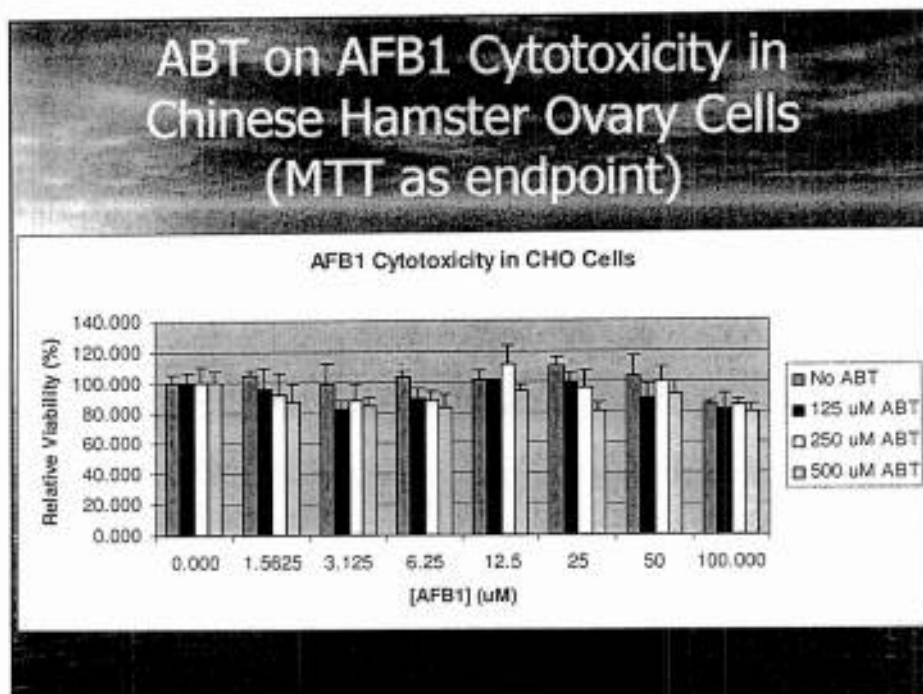
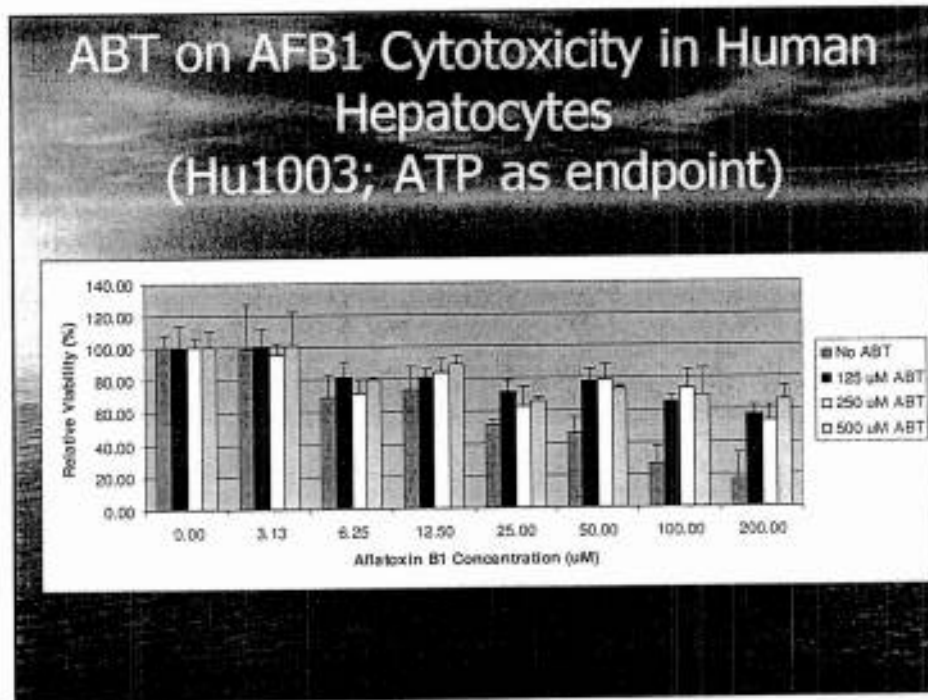
- Hour 0: Thaw and plate hepatocytes (collagen-coated 96-well plate; 10,000 cells/well)
- Hour 4: Change medium to treatment medium containing toxicants with or without P450 inhibitors
- Hour 16 (12-hr treatment) : Evaluation of cytotoxicity (ATP; MTT; GSH etc.)

## 1-Aminobenzotriazole (nonspecific)



- Azo dye
- Potent, nonspecific, mechanism-based inhibitor of P450
- Effective both in vitro and in vivo
- No apparent effects on Pgp





## CMPIA POC 1 with ABT

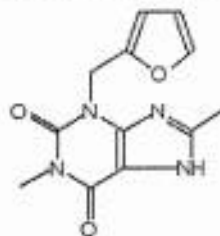
The nonspecific P450 inhibitor, ABT, caused dose-dependent reversal of AFB1 cytotoxicity, therefore confirming that metabolism is required for AFB1 hepatotoxicity

## CMPIA: Further POC

Question: Which P450 isoforms are responsible for the activation of AFB1 to cytotoxic metabolites?

Approach: Evaluate effects of isoform-specific inhibitors on cytotoxicity of AFB1 in human hepatocytes

## Furafylline



- 1,8-dimethyl-3-(2'-furfuryl)methylxanthine
- Long-acting replacement for theophylline in the treatment of asthma.
- A potent, highly specific, non-competitive, mechanism-based inhibitor of CYP1A2

## Sulfaphenazole



1. 4-amino-N-(2-phenylpyrazol-3-yl)benzenesulfonamide
2. Sulfonamide antimicrobial compound
3. Blocks pro-inflammatory and atherogenic effects of linoleic acid (increase in oxidative stress and activation of AP-1) mediated by CYP2C9
4. Selective Inhibitor of CYP2C9

## Quinidine

1. Antimalarial: Intraerythrocytic schizonticide for *Plasmodium malariae*
2. Antiarrhythmic (potassium channel blocker) acting on cardiac muscle and in Purkinje fibers
3. Potent, highly selective, competitive inhibitor of CYP2D6

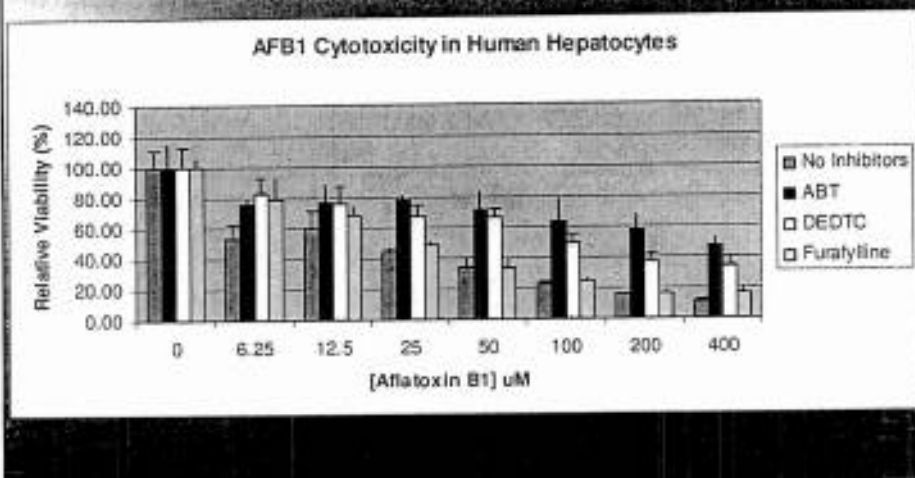
## Diethyldithiocarbamate

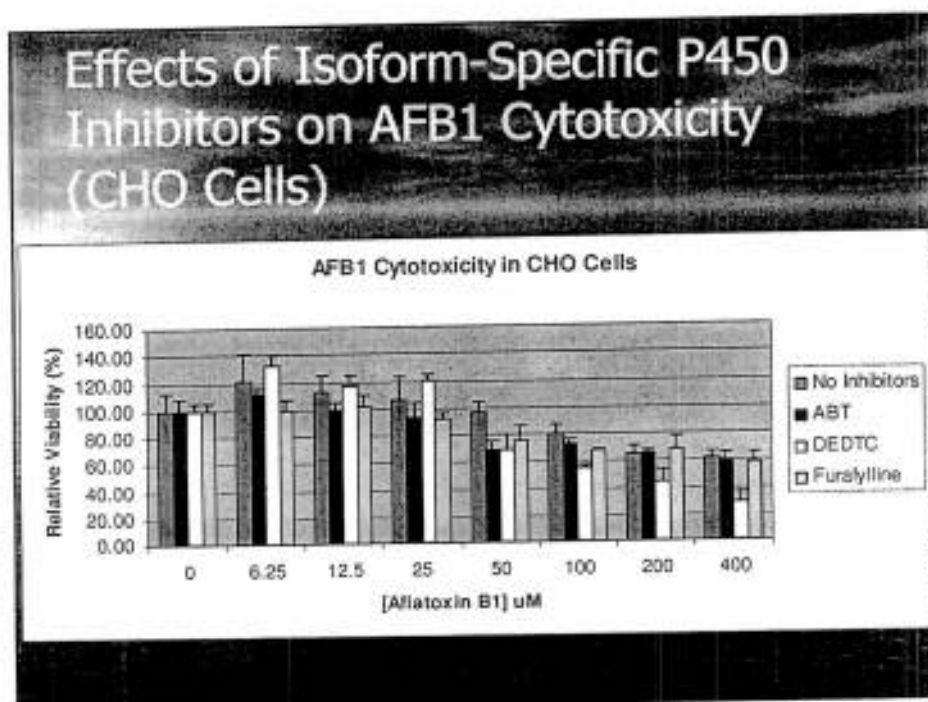
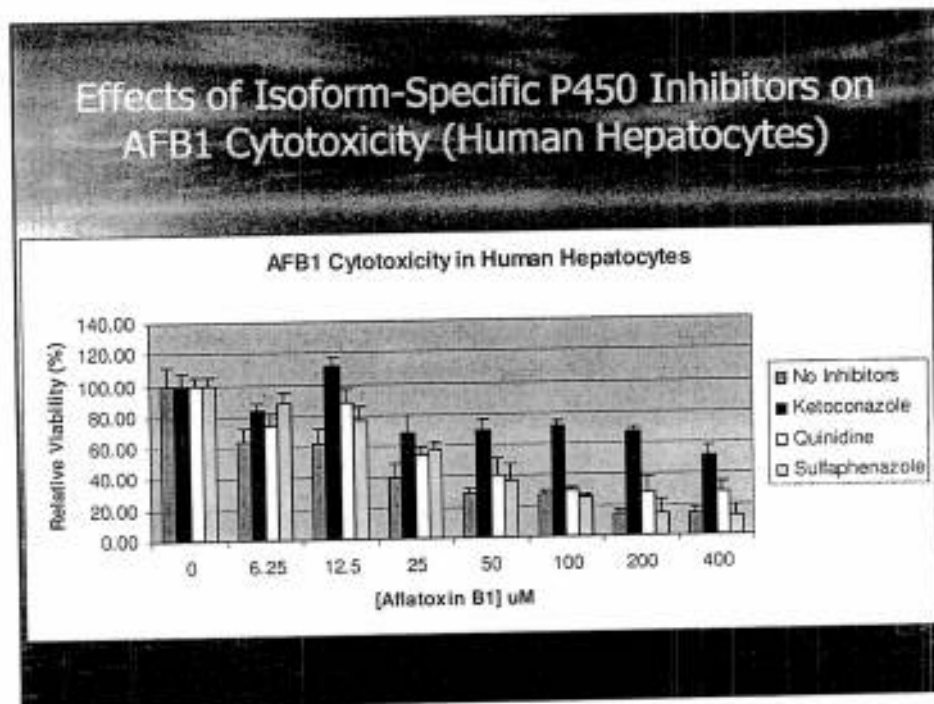
1. Pesticide, fungicide and chelating agent (mercury, copper, nickel and zinc).
2. Evaluation of T-cell deficient diseases, cancer immunotherapy, treatment of acute nickel carbonyl, cadmium and thallium poisoning.
3. Latex accelerator in rubber processing and as a chemical intermediate in the production of other diethyldithiocarbamate metal salts, such as zinc selenium and tellurium salts.
4. Active metabolite of disulfiram
5. Mechanism-based inhibitor of CYP2E1

## Ketoconazole

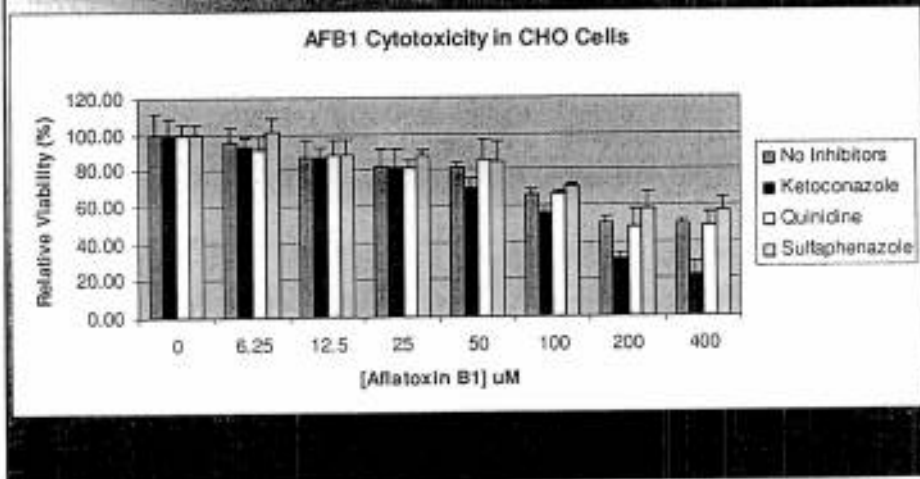
1. (±)-cis-1-Acetyl-4-[(2-[2,4-dichlorophenyl]-2-[1H-imidazol-1-ylmethyl]-1,3-dioxolan-4-yl)-methoxy]phenyl)piperazine
2. Antifungal agent
3. Competitive inhibitor of CYP3A4

## Effects of Isoform-Specific P450 Inhibitors on AFB1 Cytotoxicity (Human Hepatocytes)





## Effects of Isoform-Specific P450 Inhibitors on AFB1 Cytotoxicity (CHO Cells)



## CMPIA: Results with isoform-specific P450 inhibitors

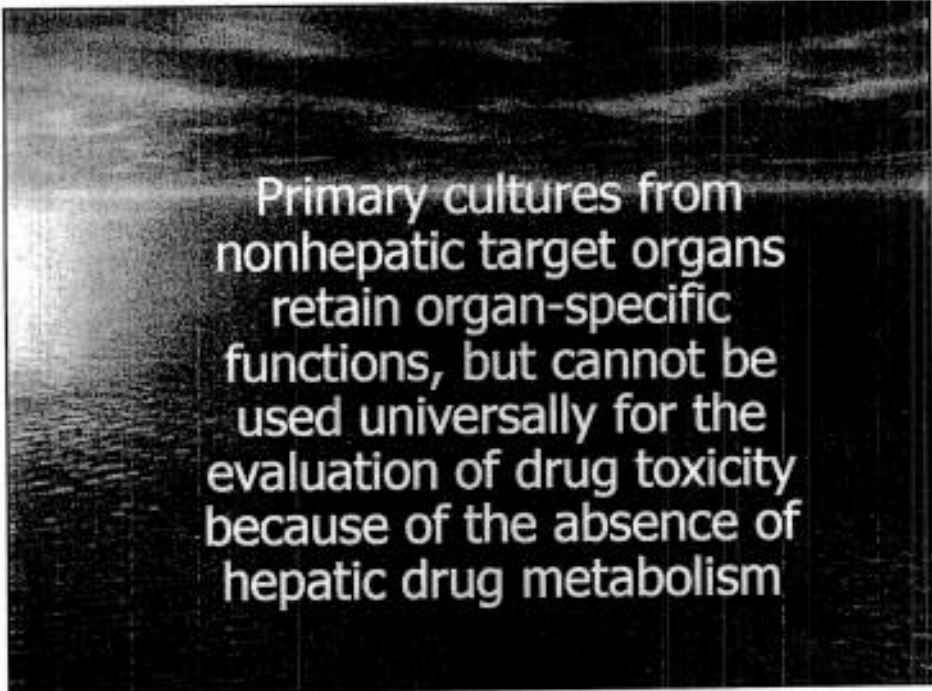
DEDTC and Ketoconazole selectively reverses the cytotoxicity of AFB1, suggesting that CYP2E1 and CYP3A4 are the key isoforms responsible for the metabolic activation of AFB1 to hepatotoxic metabolites

## Hepatotoxicity is not the only Adverse Drug Effects

- Nephrotoxicity
- Bone marrow toxicity
- Cardiovascular toxicity
- Neurotoxicity
- Skeletal muscle toxicity

## Primary cells successfully cultured from human organs

- Hepatocytes
- Endothelial cells
- Kidney tubule cells
- Osteoblasts/osteoclasts
- Astrocytes
- Airway epithelial cells
- Bone marrow cells/lymphocytes



Primary cultures from nonhepatic target organs retain organ-specific functions, but cannot be used universally for the evaluation of drug toxicity because of the absence of hepatic drug metabolism



## Role of Hepatic Metabolism for Toxicants of Extrahepatic Tissues

Formation of toxic metabolites that can be transported to distant target tissues

- Metabolic detoxification

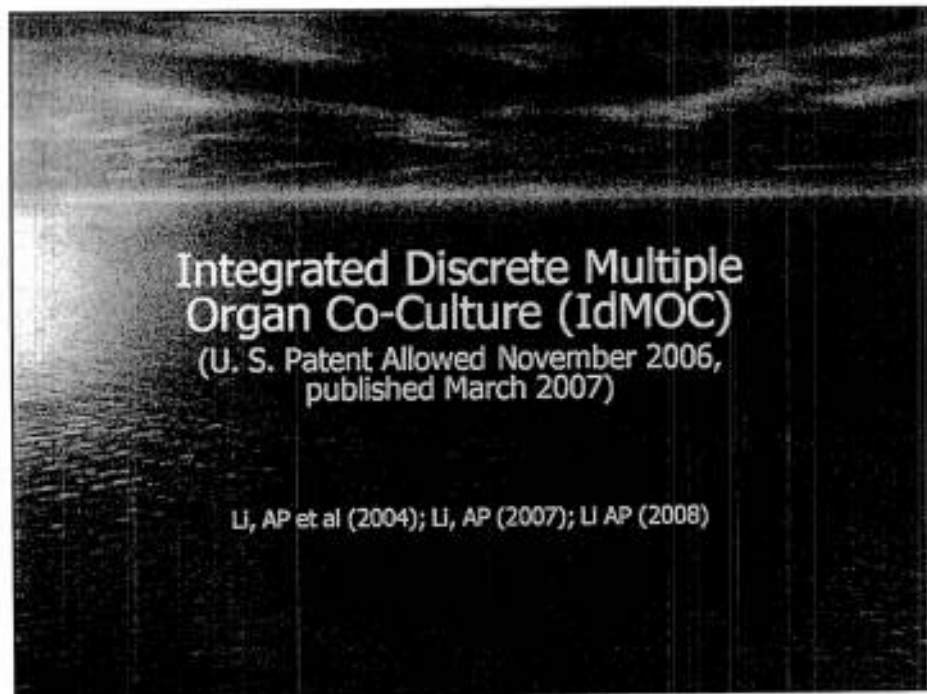
## Lack of Multiple Organ Interactions as a Major Deficiency of In Vitro Experimental Systems

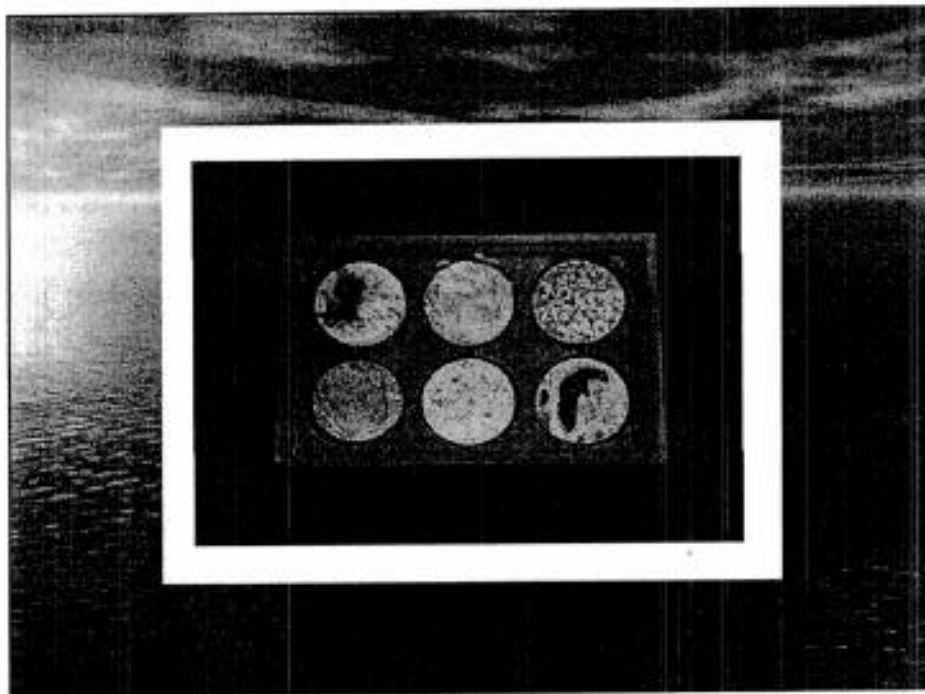
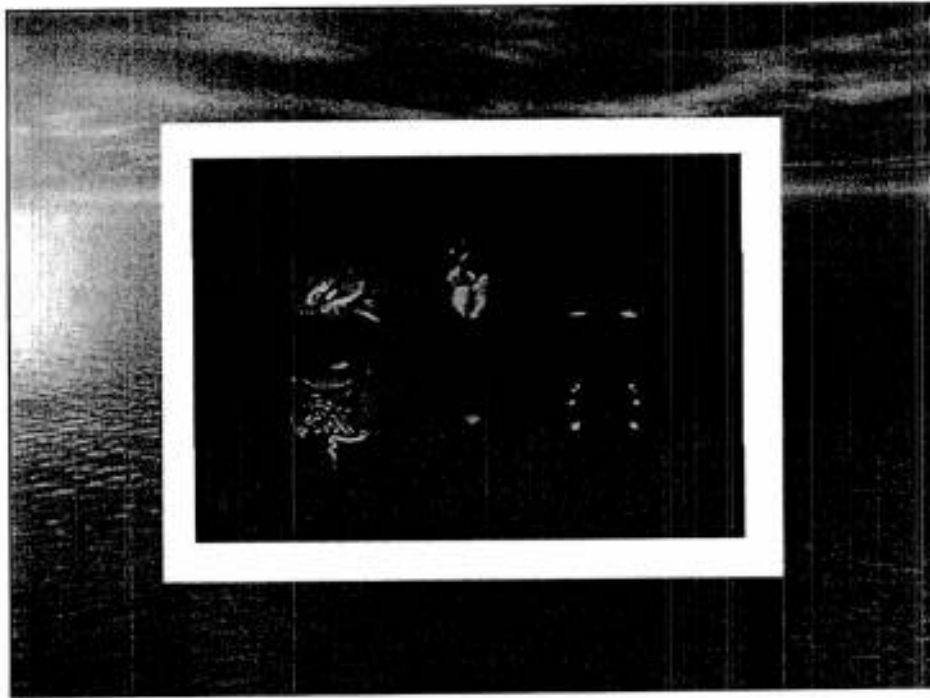
Multiple organ interactions can be key to drug toxicity

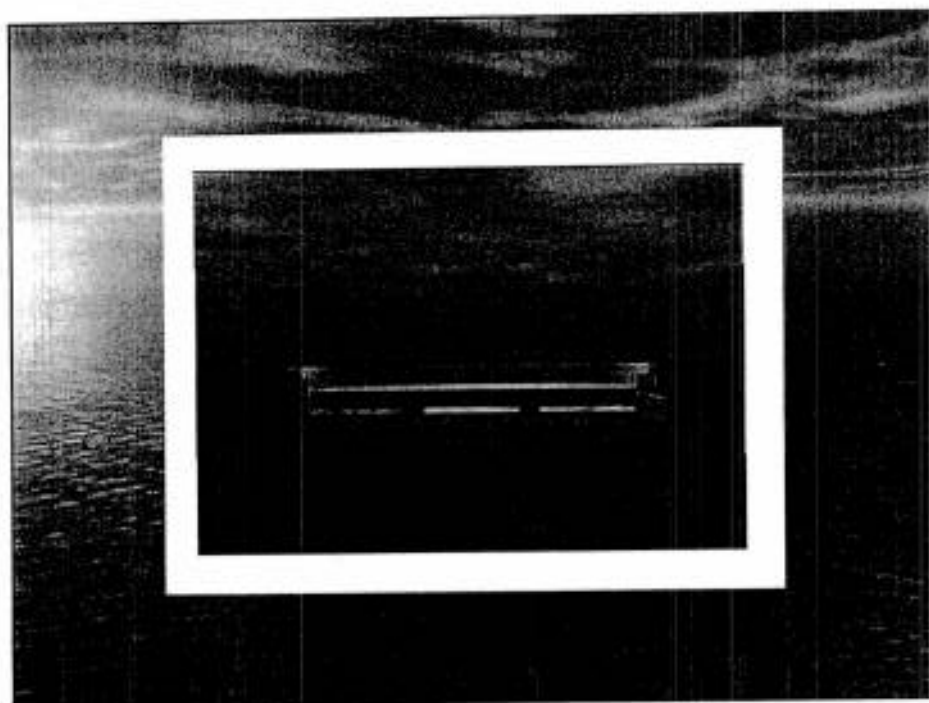
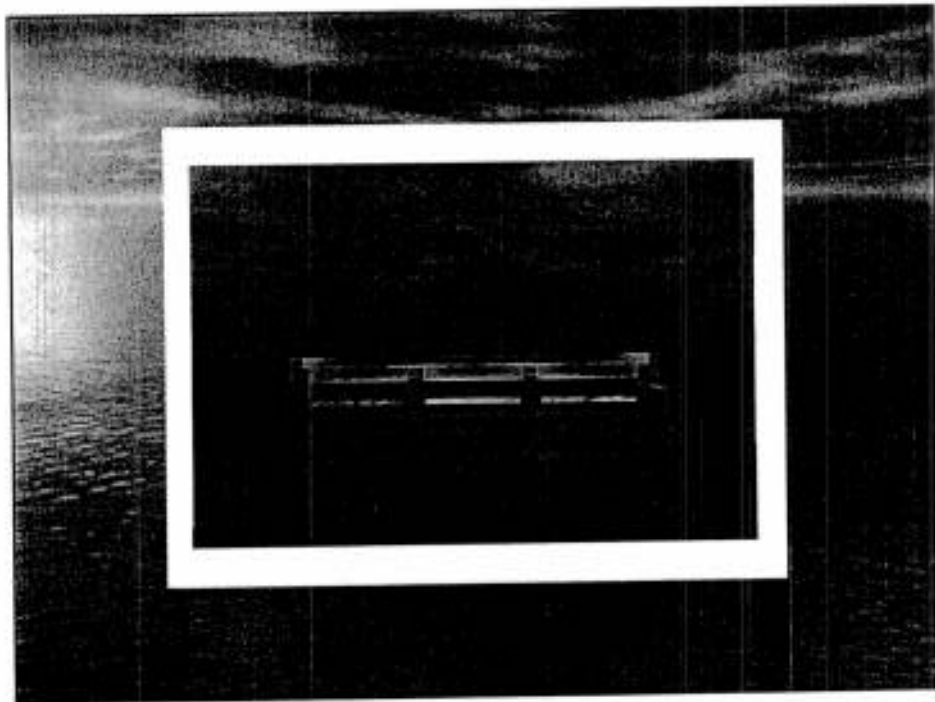
- A drug may be biotransformed by multiple organs
- A drug and its metabolites may have multiple organ effects
- Metabolites from one organ may have effects on other organ(s)

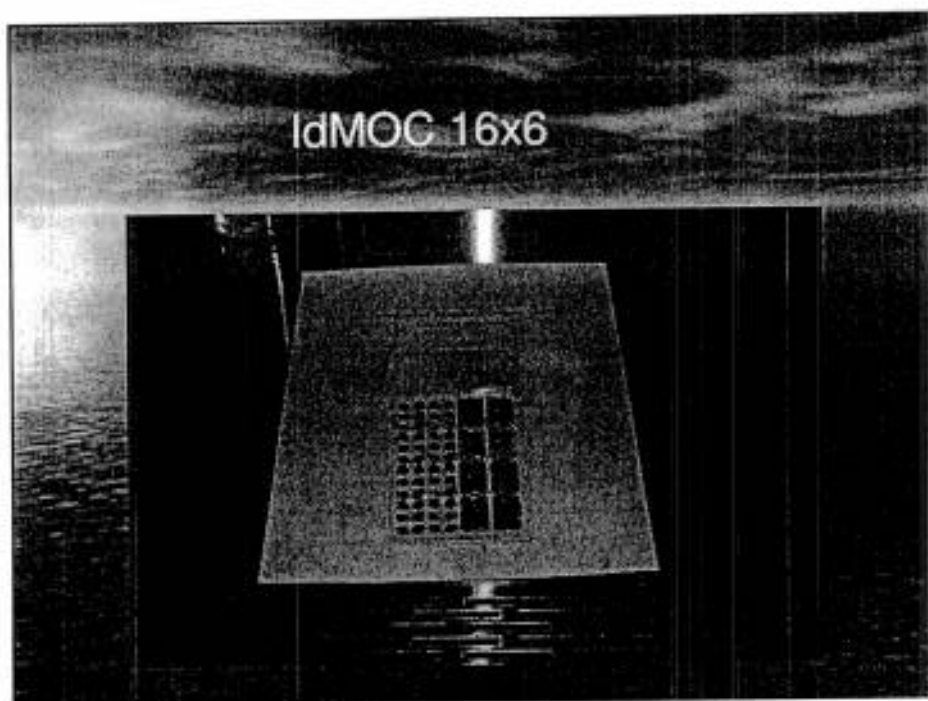
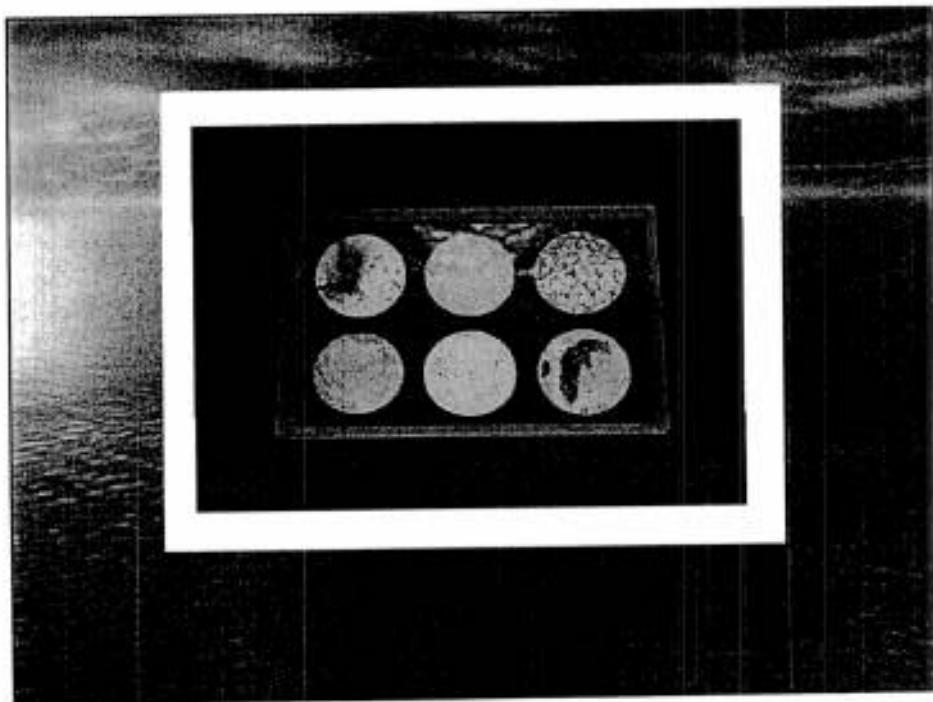
## An Ideal *In Vitro* System for Human Toxicity Evaluation

Human hepatic metabolism  
Human target organs  
Multiple organ interactions

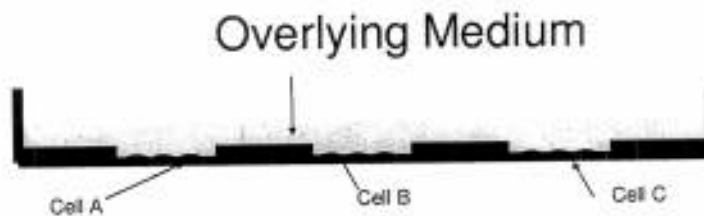








## Schematic Diagram for IdMOC



## IdMOC evaluation of organ-specific toxicity

Aflatoxin B1: Hepatotoxicant and carcinogen

## IdMOC Chamber Design (for EPA Toxcast Program)

RPTEC

Hepatocytes

SAEC

SAEC

Hepatocytes

RPTEC

RPTEC: Renal proximal tubule cells, human  
SAEC: Small airway epithelial cells, human  
Hepatocytes: Plateable cryopreserved human hepatocytes

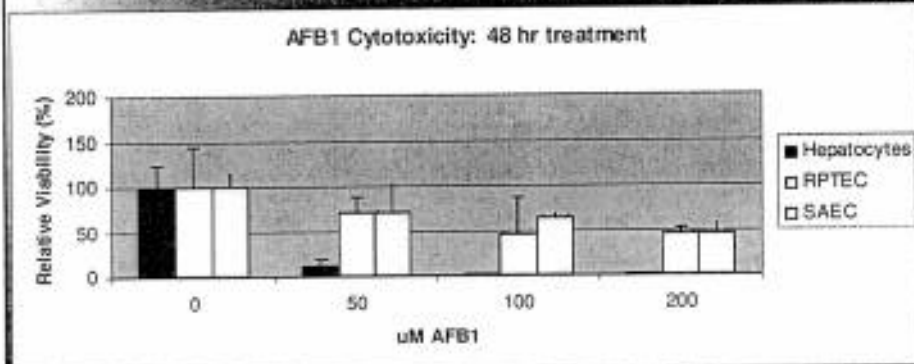
## Experimental Procedures

- Day 0: Plated cells in IdMOC
- Day 1: Treatment with AFB1
- Day 3: Measured viability using ATP as endpoint

## Relative Viability

$$\text{Relative Viability (\%)} = \frac{\text{ATP (Treatment)}}{\text{ATP (Solvent Control)}}$$

## AFB1 Cytotoxicity in IdMOC: 48 hr treatment



## IdMOC Evaluation of EPA ToxCast Chemicals

IdMOC: Human hepatocytes, human proximal tubule cells, human small airway epithelial cells

Over 300 chemicals evaluated at a single dose

Over 150 chemicals evaluated at multiple dose levels (0, 0.3, 0.8, 2.5, 7.4, 22.2, 66.7, 200  $\mu$ M), with and without P450 inhibitor ABT (500  $\mu$ M)

- Treatment duration: 48 hours
- ATP as endpoint

## Examples of Toxcast Chemical Results (EC50, $\mu$ M): Highly Toxic in All Cell Types

Chemical Name	HPT-No	HPT-Yes	RPT-No	RPT-Yes	SAE-No	SAE-Yes
Fentin	0.27	0.27	0.27	0.27	0.27	0.27
Chlorpyrifos oxon	0.27	0.10	0.27	0.27	0.27	0.27
Chlorothalonil	0.29	0.78	0.27	0.43	0.43	0.85
Niclosamide	0.39	0.36	0.32	0.39	0.48	0.71
Fluazinam	2.19	1.44	1.21	1.44	2.00	2.18
TCMTB	3.23	1.98	3.26	2.42	5.08	3.88
Emamectin benzoate	4.73	2.50	4.98	2.45	2.69	1.72
Abamectin	4.86	6.04	5.47	7.52	4.03	5.64

Chemicals highly toxic in IdMOC are also highly toxic when administered to systemic circulation\*

Fentin: LD50 i.p.  $\leq 10$  mg/kg in mouse, rat, guinea pig, rabbit.

Chlorphyrifos oxon: high active metabolite of chlorphyrifos (3000x more neurotoxic)

- Chlorothalonil: LC50 i. p. 2.5 mg/kg in mouse

\* Most are substantially less toxic upon oral administration, presumably due to low bioavailability

### Examples of Toxcast Chemical Results (EC50, $\mu$ M): Selectively Cytotoxic in Hepatocytes

Chemical Name	TVCode	HPT-No	HPT-Yes	RPT-No	RPT-Yes	SAE-No	SAE-Yes
(AFB1)	AFB1	0.92	12.80	13.50	119.00	201.00	54.60
Bifenazate	TV000017	2.52	42.10	200.00	200.00	172.00	201.00
Fenhexamid	TV000145	4.49	4.21	135.00	90.20	99.70	99.60
Pyrelostrobin	TV000055	7.36	1.40	201.00	25.50	201.00	186.00
Difenoquat metilsulfate	TV000244	7.68	7.34	105.00	74.60	200.00	200.00
Pyridaben	TV000133	16.10	0.27	149.00	200.00	48.50	74.40
Bisphenol A	TV000003	16.10	6.35	180.00	87.40	109.00	97.20

## Compounds selectively toxic to hepatocytes are hepatotoxic in vivo

AFB1: human hepatotoxicant

Bifenazate: Hepatotoxic in rats (90 day feeding study, 27.7 mg/kg/day)

Pyraclostrobin: Increased liver/body weight ratio (no effects on lung, kidney) in mouse 3-month feeding study

## Examples of Toxcast Chemical Results (EC50, uM): Selectively Cytotoxic in Renal Proximal Tubule Cells

Chemical Name	TVCode	HPT-No	HPT-Yes	RPT-No	RPT-Yes	SAE-No	SAE-Yes
Benomyl	TV000019	75.20	75.00	0.90	31.60	46.80	97.30
Cyazofamid	TV000057	109.00	2.87	6.10	6.30	114.00	15.50
Methoxychlor	TV000075	155.00	144.00	48.50	136.00	123.00	86.60

### Examples of Toxcast Chemical Results (EC50, uM): Selectively Cytotoxic in Small Airway Epithelial Cells (SAEC)

Chemical_Name	TVCode	HPT- No	HPT- Yes	RPT- No	RPT- Yes	SAE- No	SAE- Yes
Lindane	TV000010	145.00	118.00	201.00	200.00	1.17	158.00
Thidiazuron	TV000120	47.20	73.30	136.00	142.00	1.18	113.00
Parathion-methyl	TV000081	50.50	65.60	200.00	151.00	27.40	57.90
Azoxystrobin	TV000144	20.40	16.50	200.00	201.00	15.30	17.00

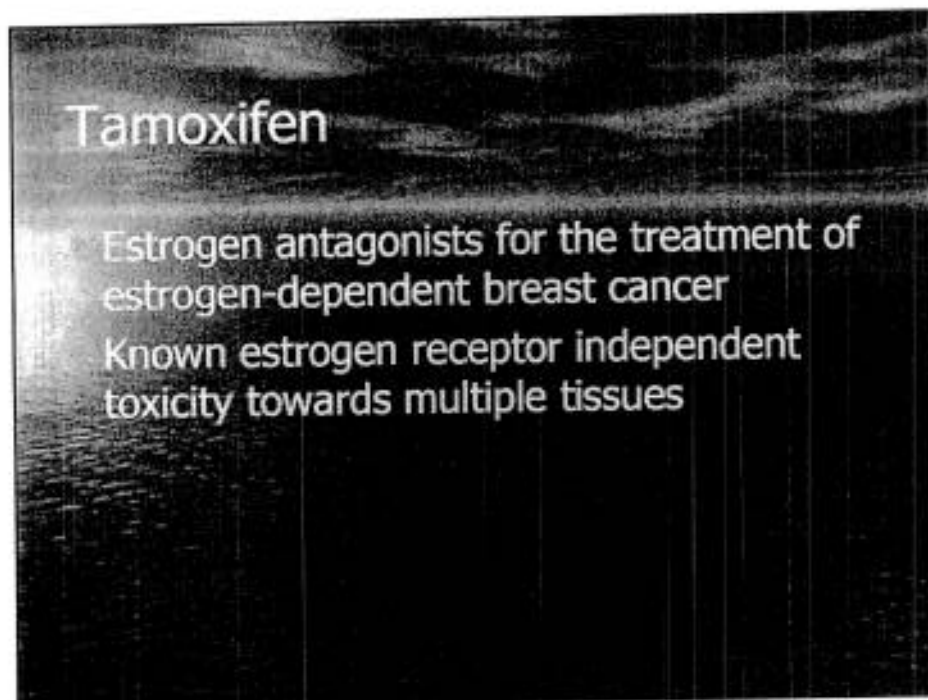
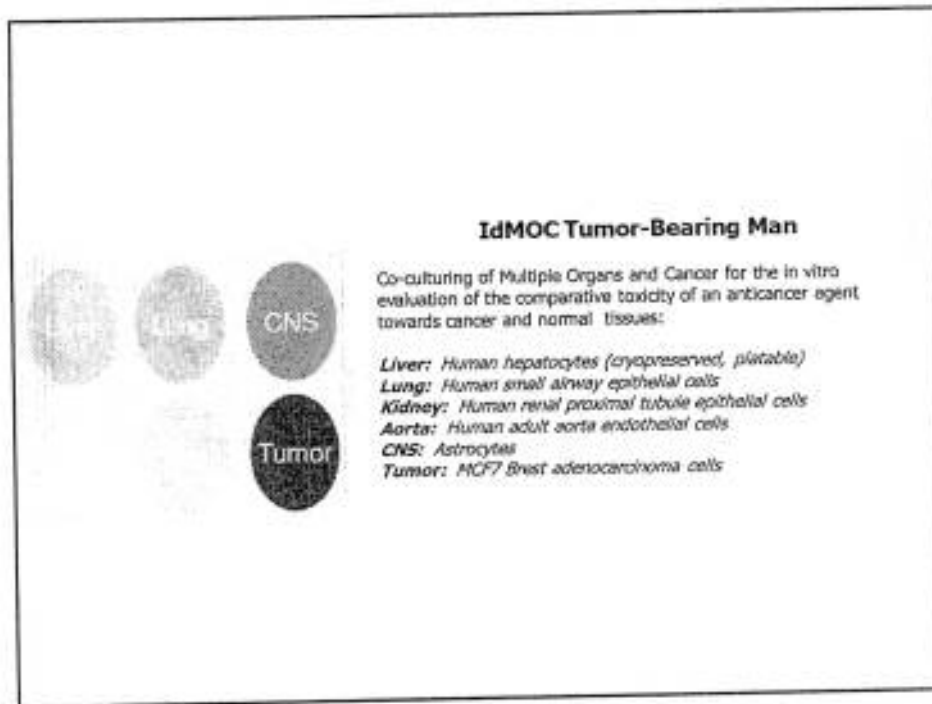
### Examples of Toxcast Chemical Results (EC50, uM): ABT Decreases Cytotoxicity

Chemical_Name	TVCode	HPT- No	HPT- Yes	RPT- No	RPT- Yes	SAE- No	SAE- Yes
(AFB1)	AFB1	0.92	12.80	13.50	119.00	201.00	54.50
Propargite	TV000389	0.99	12.50	11.20	10.70	1.20	1.43
Bifenazate	TV000017	2.52	42.10	200.00	200.00	172.00	201.00
Fluoxastrobin	TV000051	10.20	79.30	201.00	104.00	10.80	176.00
Hexythiazox	TV000371	39.20	113.00	200.00	190.00	200.00	155.00
Ethofumesate	TV000210	73.60	201.00	200.00	200.00	200.00	200.00
(Z,E)- Fenpyroximate	TV000142	89.80	200.00	201.00	200.00	63.30	200.00

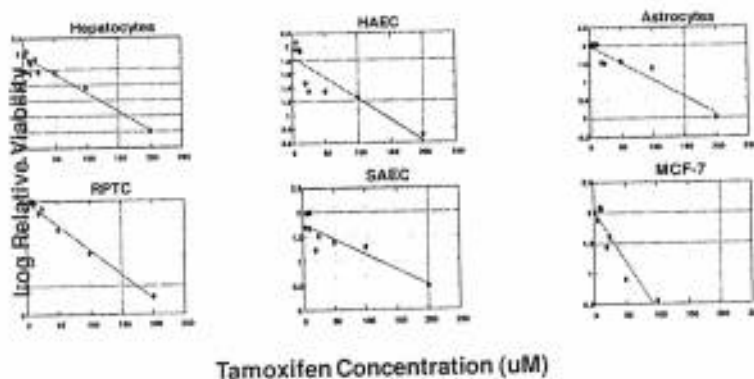
### Examples of Toxcast Chemical Results (EC50, uM): ABT Increases Cytotoxicity

Chemical_Name	TVCode	HPT- No	HPT- Yes	RPT- No	RPT- Yes	SAE- No	SAE- Yes
Cyazofamid	TV000057	109.00	2.87	6.10	6.30	114.00	15.50
Fenoxycarb	TV000062	100.00	20.60	200.00	185.00	172.00	200.00
Etoxazole	TV000286	105.00	33.60	82.70	134.00	52.20	94.10
Disulfoton	TV000105	178.00	34.40	200.00	200.00	200.00	1.46
Flufenacet	TV000146	140.00	41.00	200.00	201.00	186.00	169.00
Mancozeb	TV000367	201.00	71.70	168.00	200.00	200.00	200.00

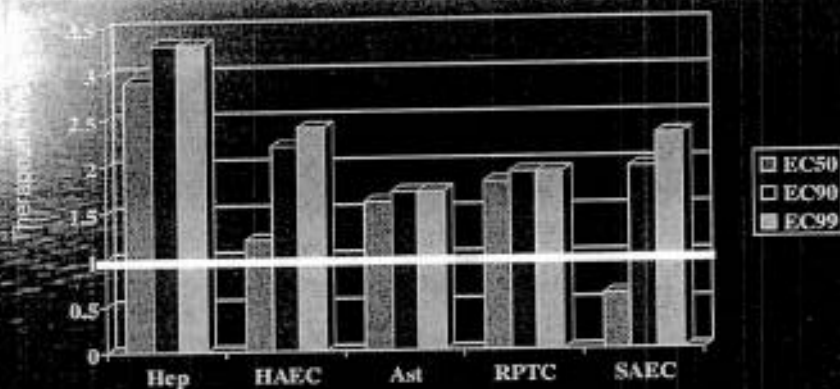
### IdMOC Evaluation of Anticancer Agents



## IdMOC Evaluation of Tamoxifen Cytotoxicity



## In Vitro Therapeutic Index Values for the Multiple Cell Types



## Application of IdMOC in Discovery of Anticancer Compounds

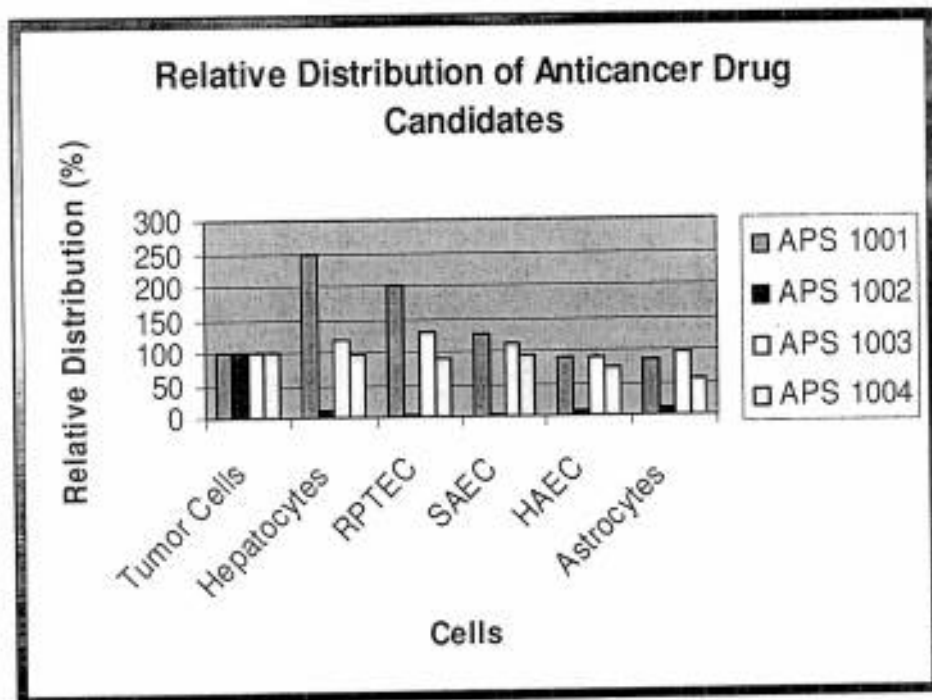
### IdMOC Drug Distribution Assay

*Rationale:*

Screen for anticancer agents which would selective target tumor cells

*Procedures:*

1. Culture the multiple cell types in IdMOC
2. Replace culture medium with Krebs Hensleit Buffer (KHB) containing drug candidates at the desirable concentrations to be evaluated
3. Incubate at 37 deg. C for 2 hours
4. Rinse IdMOC wells with ice-cold KHB
5. Add 1 N NaOH to lyse cells
6. Assay for protein and drug concentration from cell lysate

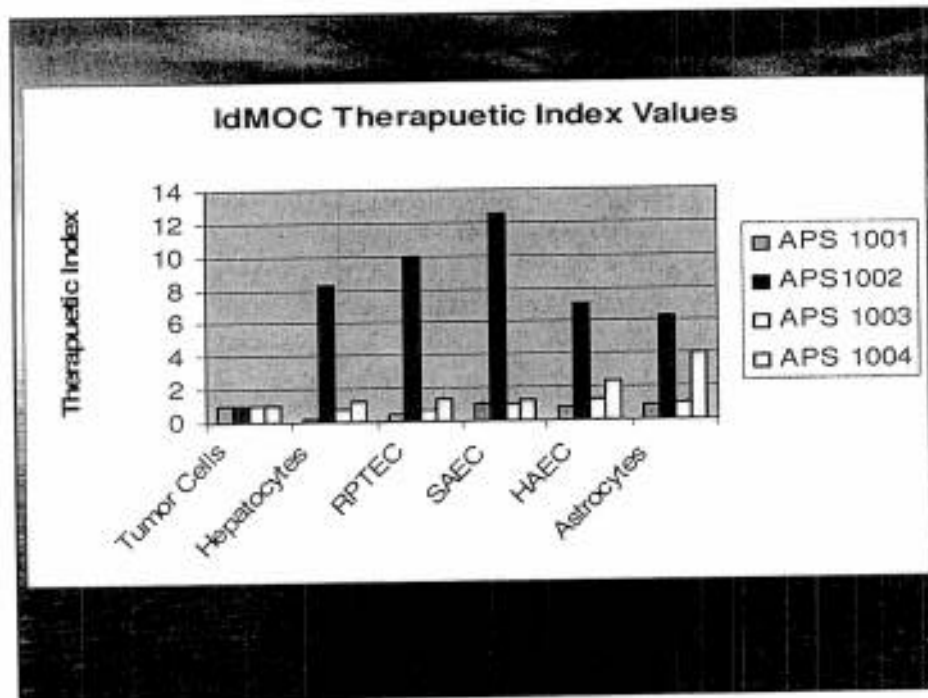


**IdMOC in vitro Therapeutic Index (TI) Assay**

**Procedures:**

1. Day 0: Plate cells in IdMOC
2. Day 1: Treat cells with drug candidates
3. Day 2: Evaluate cell viability (ATP)
4. Calculate therapeutic Index (T. I.):  

$$TI = EC50 \text{ (normal cells)} / EC50 \text{ (tumor cells)}$$



**Conclusion:**

APS 1002 is selective toxic towards tumor cells, suggesting that it may be a nontoxic anticancer agent. The results with cytotoxicity are consistent with the differential affinity of APS 1002 towards tumor cells as found with the IdMOC Drug Distribution Assay.

## Summary: IdMOC

Quantitative data can be obtained on the effects of a toxicant on cells from multiple organs or multiple cell types from a single organ

Differential cytotoxicity to specific cell types can be detected

Metabolism-related toxicity can be evaluated as illustrated by results with ABT treatment

- IdMOC-tumor bearing man represent an effective experimental system to aid the identification of anticancer compounds with minimum toxicity to normal cells

## IdMOC: An Universal Tool for Drug Discovery and Development

Co-culturing of primary cells from multiple organs with a common overlying medium, thereby modeling an organism (e.g. human) with multiple organs sharing a common body fluid

- Discrete cultures allowing the evaluation of organ-specific effects
- Interconnected culture allowing multiple organ metabolism
- Can be applied towards most disciplines of drug development, including metabolism, distribution, toxicity, and efficacy
- IdMOC as a tumor-bearing man: an effective tool for the discovery of anticancer drugs

## IdMOC Applications

- Modeling of whole organism
- Modeling of single organs
- Evaluation of multiple organ metabolism
- Evaluation of drug distribution
- Evaluation of multiple organ/cell type toxicity

## ADE screening for early drug development

- CYP3A4 inhibition, CYP3A4 induction, and hepatocyte cytotoxicity identified as most critical ADE
  - Higher throughput assays
- CMPIA for identification of metabolism-based cytotoxicity
- IdMOC for the evaluation of multiple organ toxicity

## IVAL/BRiVAL Contract Research Services

### Non-GLP screening assays: IVAL

- In vitro adverse drug effects screening for early drug development
  - Quick turn-over (1-2 weeks from chemical receipt to report)
  - Flexible

### • GLP In vitro ADME Studies: BRiVAL (BRI/IVAL)

- Definitive FDA regulatory studies
  - Standardized, validated studies
  - BRI Analytical chemistry expertise
  - IVAL ADME expertise

## Contact Information

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